SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

pH AND pH GRADIENTS REGULATE THE H_V1 ION CHANNEL IN MYELOID-DERIVED SUPPRESSOR CELLS AND MODULATE THE PHARMACOLOGY OF THE $K_{Ca}3.1$ CHANNEL OF T CELLS

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1. INTRODUCTION

1.1 Ion channels

1.1.1 General information about K^+ channels

Ion channels are membrane proteins localized either in the plasma membrane or in the membrane of several intracellular organelles. As their name suggests, ion channels let ions passively diffuse across the membrane following their electrochemical gradient. Ion channels can transport up to 10^8 ions per second across the membrane. K^+ channels belong to one of the biggest families of ion channels in the mammalian class, with almost 80 genes and even more functional proteins. They are multimers composed of different subunits, usually a pore-forming α-subunit accompanied by smaller accessory (β) subunits that have an influence on their gating and expression.

1.1.2 K_{Ca}: Ca^{2+}-activated K^+ channels

Ca^{2+}-activated K^+ channels (K_{Ca}) can either be small- and intermediate-conductance channels (SK1-3, also known as $K_{Ca}2.1$, $K_{Ca}2.2$, $K_{Ca}2.3$ and IK1, also known as $K_{Ca}3.1$) or big/large-conductance channels (BK, also known as $K_{Ca}1.1$). While $K_{Ca}2.x$ and $K_{Ca}3.1$ have a conductance of 4-14 and 32-39 pS, respectively, the conductance of BK channels is 200-300 pS. In contrast to SK and IK, BK channels are structurally different, as stated before. Besides the differences in the number of transmembrane segments (TMs), BK channels are also voltage-gated due to the charged voltage sensor domain (VSD) and they do not need auxiliary proteins to confer Ca^{2+}-dependent gating since they are directly activated by intracellular Ca^{2+} through their "regulator for conductance of potassium" (RCK) domain located in the C-terminal. Conversely, the gating of SK1-3, whose similarity lies around 80-90% and IK1, whose sequence is ~40% identical to $K_{Ca}2.x$ channels, is completely insensitive to the membrane potential. These channels need Calmodulin (CaM), a cytosolic Ca^{2+}-binding protein to confer the Ca^{2+} sensitivity of gating.

1.1.3 The structure of the SK and IK Ca^{2+}-activated K^+ channels

Cryo-electron microscopy (cryo-EM) recently unveiled the details about the precise structure of the human $K_{Ca}3.1$. The members of this sub-family assemble in tetrameric structures and each subunit has 6 TMs, with the pore-forming domain (PD) constituted by the sequence TM5-P-TM6. The pore-loop (P) is located between TM5 and TM6 and TM1-4 directly contacts the PD of the same subunit. Both the N- and the C-terminal are located in the intracellular side. The C-terminus is preceded by three α-helices (HA, HB and HC): while the first two helices run almost parallel to the membrane and anti-parallel to each other, HC takes a 90° turn and, coupled with the other HC domains from

the other three subunits, creates a perpendicular column at the bottom of the channel. $K_{Ca}2.x$ and $K_{Ca}3.1$ channels are unable to sense directly the intracellular Ca^{2+}, hence they associate intracellularly with four CaM (one per each subunit), a cytosolic protein with two conserved globular lobes connected by a very flexible linker. CaM binds constitutively HA and HB with its C-lobe, whereas the N-lobe has more freedom of movement. Binding up to 4 Ca^{2+} ions prompts CaM to reach an extended conformation.

In $K_{Ca}3.1$ the intracellular linker between TM4 and TM5 is formed by two different α-helices ($TM_{4-5}A$ and $TM_{4-5}B$) which have contacts both with HA and the pore region of the same subunit, in particular TM6. Cryo-EM has shown that $TM_{4-5}A$, which is highly conserved across the SK channel family, is the portion to which the N-lobe of CaM binds when it is in the Ca^{2+}-bound extended conformation. The interaction between CaM and $TM_{4-5}A$ causes a downward pull of $TM_{4-5}B$ and the pore-lining TM6. This will result in the dilation of the cytosolic entrance to the pore from ~1 to 3.5 Å, thereby permitting the partially hydrated K^+ ions to freely flow outside of the cell. This is partially in contrast with the historical knowledge about K_{Ca} channels based on the outdated crystal structure, which proposed the N-lobe of CaM to bind the C-terminal of the adjacent subunit in order to move TM6 and open the channel.

1.1.4 Positive and negative modulators of Ca^{2+}-activated K^+ channels

Even if the members of the $K_{Ca}2.x$ and $K_{Ca}3.1$ families are structurally similar their sensitivity to positive and negative modulators are remarkably different. Among the negative modulators we can find pore-blocking peptide toxins and small organic molecule inhibitors. Besides blockers, several activators (henceforth also called enhancers) of Ca^{2+}-activated K^+ channels have been discovered. We used, as positive modulators, two molecules: SKA-31 and Riluzole. Riluzole is a neuro-protective drug used to inhibit the release of glutamate as a neurotransmitter, but it has been repurposed as an enhancer of Ca^{2+}-activated K^+ channels. SKA-31 is one of the molecules obtained using Riluzole as a template, but it is a much stronger enhancer (for $hK_{Ca}3.1$ the EC_{50} are 1-2 μM for Riluzole and 220-260 nM for SKA-31).

1.1.5 The mechanism of action of the positive modulators of Ca^{2+}-activated K^+ channels

Most enhancers are lipophilic substances, therefore they are able to cross the plasma membrane reaching the intracellular regions of the Ca^{2+}-activated K^+ channels. Lee and MacKinnon proposed that the binding pocket is between $TM_{4-5}A$ and the N-lobe of CaM. It is believed that the involved residues could be L185, S181 and A184. Interestingly, even if the cryo-EM structure revolutionized the knowledge about what part of $K_{Ca}3.1$ is actively implicated in the recognition of a substance like 1-EBIO, the residues of the N-lobe of CaM that bind the positive modulators are still thought to be essentially

the same (F19, I27, L32, M51, I52, E54, V55, I63, F68, M71, M72, K75). Constant progress has been made in regard to understanding how the modulators and the channels interact, but it is still not clear what exactly ensues after this interaction. $K_{Ca}3.1$ and the $K_{Ca}2.x$ channels have been reported to have submicromolar Ca^{2+} sensitivity ($K_{Ca}3.1$ EC_{50}: 100-400 nM, $K_{Ca}2.x$: 300-750 nM), this means that the physiological intracellular concentration (~100 nM) is normally unable to strongly activate the channels. Activators enhance the overall Ca^{2+} sensitivity of the channels so that their open probability increases dramatically even without changing the intracellular Ca^{2+} concentration. How this exactly happens is still not clear. Specific aminoacidic mutations can also positively alter the Ca^{2+} sensitivity of channels like $K_{Ca}2.3$ and get the same effect that would be obtained via the use of the activators.

1.1.6 H^+ channels: structure and general characteristics

Proton currents were recorded since the '70s, however, the gene encoding the voltage-gated H^+ channel (H_v1) was discovered only in 2006. H_v1 is not redundant and only one gene has been recognized in every species in which it has been discovered. Because of the extremely low amount of free H^+ in the intracellular compartment (40-70 nM), this ion channel needs to be extremely specific and selective and, to date, it has not been proven that H_v1 can let other ions pass across the membrane other than H^+. The strikingly perfect selectivity of H_v1 is accompanied by a peculiar structure: while most voltage-gated channels have a region (usually comprising TM5 and TM6) that serves as a pore-forming domain and a selectivity filter, H_v1 has only 4 TMs out of the 6 normally present and they constitute both the voltage sensor and the permeation pathway for H^+. While functional as a monomer, *H_v1* is usually expressed as a *dimer*. The 4 TMs of each protomer organize themselves as an hourglass with a narrow constriction in the middle capable of selecting only for H^+ ions thanks to the residue Asp^{112}, implying that each protomer has its own conduction pathway. As the name "H_v1" states, the proton channel is voltage-sensitive and the positive charges (3 Arg residues) important for voltage sensing are in TM4. A unique feature of this channel is that the TM4 of H_v1 is extremely sensitive to ΔpH ($pH_o - pH_i$) more than to pH itself. The threshold voltage when is around 0 mV when $\Delta pH \sim 1$, but it becomes +40 mV when $\Delta pH \sim 0$. In any case the H^+ currents through H_v1 have slow activation kinetics and take several seconds to develop fully. Changing pH_i while keeping pH_o constant (hence lowering ΔpH) caused a movement in the TM4. This movement did not happen if ΔpH was kept constant, i.e. changing pH_o as well, proving that TM4 is able to sense the difference between the pH outside and the pH inside, but does not perceive the absolute change either of the intracellular or the extracellular pH. pH itself influences the activation and deactivation kinetics of the channel. In particular, the ON-gating current decay time constants depend on the internal and external pH values instead of ΔpH. This could be explained by the dependence of the conformation of certain portions of the protein, e.g. TM4, on both pH_i and pH_e.

H_v1 is mostly expressed in cells belonging to the immune system. The major role of H_v1 in these cells is to contribute to the "respiratory burst", id est the production of several highly reactive oxygen species (ROS) to destroy foreign bodies. The accumulation of H^+ will inevitably lead to the acidification of the cytoplasm from a value of 7.4 to ~6 in as low as 10 minutes. H_v1 channels, aided by the Na^+/H^+ antiport exchangers, have the leading role of removing the excess of H^+.

1.1.7 Inhibitors of H_v1

Because of its odd structure and the relative novelty of its discovery, specific blockers of this channel have not been discovered yet. Classical inhibitors of H_v1 are divalent metal ions like Zn^{2+} and Cu^2. Recently the family of guanidine derivatives have been identified as H_v1 blockers as well. 5-chloro-2-guanidinobenzimidazole (ClGBI), one of the most frequently used substances to pharmacologically demonstrate the presence of functional H_v1 ion channels, is able to cross the membrane and inhibits hH_v1 with a K_d of ~20 μM. It must be acknowledged, however, that ClGBI is a "dirty drug", id est, it displays low selectivity among various voltage-gated ion channels (e.g., $K_v1.3$ and $Na_v1.5$ are both blocked by ~μM of ClGBI).

1.2 The relationship between the immune system, cancer and ion channels

1.2.1 The role of K^+ channels in the activation of T cells

The activation of T cells is the result of an intricated phosphorylation cascade that ensues the formation of an immune synapse. Several T cell receptor -associated Tyr-kynases will phosphorylate a series of adaptor proteins activating multiple pathways. The end result is an increase in intracellular Ca^{2+} due to the ingress of extracellular Ca^{2+} and its release from the endoplasmic reticulum. Ca^{2+} will ultimately provoke, through the activation of phosphatases like calcineurin, the dephosphorylation and translocation of the transcription factor NFAT, which contributes to the activation of specific genes involved in the T cell activation. The influx of Ca^{2+} from the extracellular side must be balanced by the efflux of another positive ion: T cells use $K_v1.3$ and $K_{Ca}3.1$ to conduct outward K^+ fluxes, thereby avoiding an excessive depolarization of the plasma membrane, which would halt the Ca^{2+} ingress. The number of $K_v1.3$ and $K_{Ca}3.1$ channels on the membrane will increase when T cells get activated.

1.2.2 The immune system and the tumor

Tumor tissue can be considered a broadly inflamed structure that promotes inflammation and that, in turn, is promoted by inflammation, which is tightly bound to the immune system. We can find almost all types of infiltrating immune cells in tumor tissues,

in particular, cytotoxic $CD8^+$ T cells are almost always a favorable prognosis factor. On the other hand, myeloid cells are a family of both anti-tumor and pro-tumor elements dwelling in the tumor microenvironment (TME) and deeply influencing its evolution. Activated neutrophils and activated macrophages, in this case called tumor-associated macrophages (TAMs), usually show an anti-tumor activity but are often overshadowed by a close family of pathologically activated pro-tumor cells, mostly known as myeloid-derived suppressor cells (MDSCs), branched in monocytic-MDSCs (M-MDSCs) and polymorphonucleate-MDSCs (PMN-MDSCs).

1.2.3 Myeloid-derived suppressor cells (MDSCs)

MDSCs exert their action in several different ways, both through direct contact with other immune cells and by the production of molecules that have an impact on the tumoral landscape in which these cells live. Just like macrophages and neutrophils, MDSCs produce several ROS and reactive nitrogen species (RNS), which can cause the oxidation and nitration of specific residues of the TCR/CD8 complex present on cytotoxic T cells. MDSCs deplete extracellular L-Arginine as well as L-Tryptophan and L-Cysteine through the overexpression of the enzymes ARG-1 and IDO1/2 and this inhibits T cell proliferation. Apart from targeting anti-tumor cytotoxic T cells and dendritic cells, MDSCs can also produce cytokines and other molecules that enhance the tumor cell stemness, induce angio- and vasculogenesis and promote extravasation and metastasis. Unfortunately, the lack of specific surface biomarkers makes it difficult to distinguish, especially *in vivo*, one subtype of myeloid cells from another, hindering the research on tumor-associated MDSCs. As compared to T cells, relatively less is known about the channelome of MDSCs.

1.2.4 The positive modulators of Ca^{2+}-activated K^+ channels can counteract high K^+ and high adesonine concentration in the tumor microenvironment

Infiltrating T cells, attracted by the inflammation-related cytokines secreted in the tumor, end up in a unique environment. Rapidly growing tumors are characterized by hypoxia and nutrient deficit. Cancer cells will die in big numbers and release their intracellular content (mostly K^+ and ATP/adenosine) in the extracellular tumoral milieu contributing to the development of a chemically pro-tumoral landscape. It has been extensively shown, for example, that tumor interstitial fluid is rich mostly in K^+ ions, which directly derive from the local high necrotic rate.

1.2.5 The tumor microenvironment and acidosis

Besides the accumulation of adenosine and K^+ ions, the TME is also characterized by a severe dysfunction of the pH homeostasis. Solid cancers, in particular, because of their stiffness and rapid growth, soon face hypoxia and nutrient deprivation. Cancer cells counter this unlivable environment by switching towards a more glycolysis-

dependent metabolism and triggering the so-called "Warburg effect". The physiological extracellular pH (pH_e) of human tissues is around 7.2-7.5, but the large amount of lactate and H^+ released during the cancer growth drive it towards values of 6.5-6.9. On the other hand, the usual intracellular pH does not steer away from 6.9-7.2, but in cancer cells it can reach more alkaline values, hitting 7.3-7.6. The alteration of the extracellular pH can directly affect the behavior of T cells *in loco*. Decreasing the extracellular pH to 6.5-6.6 will temporarily impair the activation and the proliferation of both human and murine T cells.

1.2.6 The effects of acidity on K^+ channels of immune cells

Ion channels, as proteins, are sensitive to different kinds of stresses. Some of them respond to mechanical inputs, many to changes in temperature and others to pH variations. $K_v1.3$, along with other K_v channels, is very sensitive both to temperature and pH. Increasing the temperature from 5 to 42 °C has a positive effect on the conductance and the open probability of the channel and causes a dramatic acceleration in its activation, inactivation, and recovery kinetics as well. Similarly, an increase in the whole-cell current and acceleration in the activation and inactivation kinetics of $K_v1.3$ can be observed when the extracellular solution shifts from an acidic pH of ~5 to a basic pH of ~8. On the other hand, intracellular pH variations have a big impact on the amplitude of $K_v1.3$ currents, but not on their kinetics. Despite being as important as $K_v1.3$, $K_{Ca}3.1$ has been less studied and almost nothing is known about how pH_e and pH_i influence these currents. Nothing is known either about the sensitivity of its positive modulators to pH_e and pH_i. If $K_{Ca}3.1$ enhancers like Riluzole, 1-EBIO or one of the many other similar substances are intended for use to boost the anergic immune system in the tumoral milieu, it must be known whether a perturbed pH could dampen or potentiate the effect of such drugs.

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2. AIMS OF THE STUDY

2.1 Isolation and ion channel characterization of murine tumor-associated myeloid-derived suppressor cells

MDSCs are a common component of the immunosuppressive tumor microenvironment. Although the channelome to most immune cells is well characterized, the ion channel expression and the function of ion channels in MSDSc was not known. To address this, we inoculated Lewis Lung Carcinoma cells subcutaneously in mice and collected MDSCs for electrophysiological studies. Our aims were:

1. To isolate MDSCs from freshly excised tumors and categorize them into subfamilies through flow cytometry and immunofluorescence.
2. To characterize the ion channel repertoire of MDSCs subfamilies through patch-clamping, and define the biophysical (kinetics, pH sensitivity) and pharmacological properties of the measured ion currents.

2.2 Analysis of the sensitivity of $K_{Ca}3.1$ and of its positive modulators Riluzole and SKA-31 to intracellular and extracellular pH variations

$K_{Ca}3.1$ is an important ion channel in the immune system and has a role in promoting anti-cancer immunity. Moreover, several enhancers of its activity have been proposed as possible useful tools against cancer. Extra- and intracellular pH regulation is dramatically altered in cancer, however, neither the pH sensitivity of $K_{Ca}3.1$ nor that of $K_{Ca}3.1$ activators is known. To address these our aims were:

1. To characterize the sensitivity of $K_{Ca}3.1$ to intra- and extracellular pH combinations both using peripheral blood lymphocytes and transfected CHO cells.
2. To characterize the sensitivity of the positive modulators Riluzole and SKA-31 to intra- and extracellular pH combinations

3. MATERIALS AND METHODS

3.1 Cell cultures

Human venous blood from anonymized healthy donors was obtained from a blood bank. The peripheral blood lymphocytes (PBLs) were isolated through Histopaque1077 (Sigma-Aldrich Hungary, Budapest, Hungary) density gradient centrifugation. Cells obtained were resuspended in RPMI 1640 medium containing 10% fetal calf serum (Sigma-Aldrich), 100 µg/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine, seeded in a 24-well culture plate at a density of 5 x 10^5 cells per mL, and grown in a 5% CO_2 incubator at 37°C for 2–5 days. Phytohemagglutinin A (PHA, Sigma-Aldrich) was added in 5, 7 or 10 µg/mL concentrations to the medium to boost the K^+ channel expression.

Chinese hamster ovary (CHO) cells (gift from Yosef Yarden, Weizmann Institute of Science, Rehovot, Israel) were maintained by culturing in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 2 mM L-glutamine, 10% FBS, 100 µg/mL streptomycin and 100 U/mL penicillin G (Sigma-Aldrich) at a density of $0.5 - 1 \times 10^6$ cells per mL in a humidified incubator at 37°C and 5% CO_2. Cells were passaged 3 times in a week following a 2–5 min incubation in 0.05% trypsin-EDTA solution at 37°C. Cultures were used up to passage number 20. PCR-based tests were routinely used to detect mycoplasma infection, only mycoplasma-free cultures were used for experiments.

CHO cells that do not express endogenous voltage-gated ion currents were transiently transfected with the following plasmids encoding $hK_{Ca}3.1$ and turboGFP in a pCMV6-AC-GFP (OriGene Technologies) vector; H192A-$hK_{Ca}3.1$ in a pEGFP-C1 vector (a kind gift from Bernard Attali, Tel Aviv University, Israel); $hK_{Ca}2.2$ in a pCDN3 plasmid (a kind gift from Bernard Attali, Tel Aviv University, Israel) and T79D-rCaM in a pcDNA3 plasmid (a kind gift from Bernard Attali, Tel Aviv University, Israel). TurboGFP is a modified version of ppluGFP2, derived from *Pontellina plumata*, and is characterized by a fluorescence up to three times higher than EGFP. Transfections were performed using the Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The cells were grown under standard conditions. GFP-positive transfectants were identified using Nikon TMS fluorescence microscope (Nikon, Tokyo, Japan), and currents were recorded 24 to 48 h post transfection.

CH12 B cell lymphoma cells were grown in RPMI 1640 medium supplemented with 10% FBS, 1% GlutaMAX, 1% penicillin-streptomycin. The Lewis lung carcinoma cell line (LLC) was a kind gift from László Nagy (Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen, Hungary). Cells were grown in RPMI 1640 medium supplemented with 10% FBS, 1% GlutaMAX, and 1% penicillin-streptomycin.

3.2 Tumor model and cell sorting

LLC cells (3×10^6/0.1 mL PBS) were injected subcutaneously on the right flank of syngeneic 8- to 12-week-old male C57BL/10 wild-type mice. Tumors were excised and cut into small pieces in isolation buffer (RPMI 1640 medium) followed by protease digestion using enzyme mixture (collagenase I (CLSS-1, Worthington, Columbus, OH, USA, 10 U/mL), collagenase IV (CLSS-4, Worthington, 400 U/mL), and DNase I (DCLS, Worthington, 30 U/mL)) for 30 min at 37°C. Cells were filtered through a 70 µm strainer and the red blood cells were lysed in ACK (Ammonium Chloride Potassium) lysing buffer. After washing and spinning, cells were resuspended in Hanks' Balanced Salt Solution (HBSS) buffer and stained with live/dead stain (Fixable Viability Dye eFluor 506, eBiocience, San Diego, CA, USA, 1 µg/mL) in HBSS for 30 min at 4°C to exclude the dead cells. After washing, cells were preincubated in MACS buffer (phosphate buffered saline (PBS) pH 7.2, supplemented with 0.5% BSA and 2 mM EDTA) with rat anti-mouse CD16/CD32 FcγR blocking antibody (for 5 min at 4 °C and stained for 30 min at 4°C with appropriate antibodies, in order to sort the PMN-MDSCs and Mo-MDSCs, using BD FACSAria III sorter (BD Biosciences, San Hose, CA, USA). Details regarding the antibodies, flow cytometry and sorting are in the original publication as this analysis is not part of the dissertation.

3.3 Western blot

The sorted cells were lysed in 2x Laemmli buffer supplemented with proteinase (Sigma/Merck KGaA, Darmstadt, Germany, cat #P8340) and phosphatase inhibitor (Sigma, cat #P5726) cocktail followed by a denaturation step at 98°C for 5 min. Fifteen µg protein per sample was run on 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The non-specific binding sites were blocked by incubating the nitrocellulose membrane in 5% w/v non-fat dry milk in Tris-Buffered Saline (TBS) supplemented with 0.2% v/v Tween (TBST) for 1 h. Next, the nitrocellulose membrane was incubated with primary antibodies (anti-H_v1 and -β-actin) diluted in 2.5% w/v non-fat dry milk in TBST overnight at 4 °C. After washing for 3×7 min in TTBS, the nitrocellulose membrane was incubated with donkey anti-rabbit IgG-HRP linked secondary antibody for detection of H_v1 protein. Sheep anti-mouse IgG-HRP linked secondary antibody was used for the detection of actin. The chemiluminescence signal was detected using an Azure c300 Gel Imaging System (Azure Biosystems, Dublin, CA, USA). CH12 B cell lymphoma lysate was used as a positive control. The Western blot analysis was performed on sorted cells from 3 different experiments. The relative H_v1 protein level was calculated using Azure Spot Pro Analysis software and expressed as H_v1/actin ratio.

3.4 Electrophysiology and pharmacology

Electrophysiology measurements were carried out using the patch-clamp technique in voltage-clamp mode. Whole-cell currents were recorded using a Multiclamp 700B amplifier connected to a DigiData 1440A digitizer (Molecular Devices, Sunnyvale, CA, USA). Micropipettes were pulled from GC 150 F-15 borosilicate capillaries (Harvard Apparatus Kent, UK) resulting in 3 to 5 MΩ resistance in the bath solution. Current traces were lowpass-filtered through the built-in analog 4-pole Bessel filters of the amplifiers and sampling frequency was set at least twice the filter cutoff frequency. Currents are either shown as absolute values or expressed as current density obtained by dividing the currents measured in pA with the cell capacitance in pF to yield pA/pF. Recordings were carried out at room temperature (20–25°C), The patch-clamped cell was perfused with control and test solutions using a gravity-driven custom-built perfusion system, and excess bath solution was removed constantly by vacuum suction.

3.4.1 Recording whole-cell currents in murine PMN- and Mo-MDSCs

Voltage ramps (2000 ms long from −60 mV to +150 mV, every 15 s) were used to demonstrate qualitatively the dependence of the activation threshold of the H_v1 currents on various pH_e values. Traces were filtered (lowpass boxcar, 25 smoothing points), off-line leak-corrected manually point-by-point. A linear regression line was fit to the data points below the activation threshold of the H^+ current (between 50 ms to 330 ms, corresponding to −60 mV and −30 mV) and the fitted parameters were used to subtract the non-specific leak. The leak-corrected currents between +145 mV and +146 mV were extracted, averaged, and considered as the peak current. The average currents of two or three stable traces at a given pH_e condition defined one data point.

The current–voltage (I–V) relationships and the activation threshold voltage of the currents (V_{thr}) were determined using 2 s long step depolarizations from a holding potential of −80 mV to +100 mV in +10 mV increments. The protocol was applied every 15 s; the sampling rate was 5 kHz. For the I-V curves, every trace was filtered (lowpass boxcar, 25 smoothing points) and leak-corrected manually. Peak currents were calculated as the average of the last 18 points (i.e., between 2051.1 and 2051.9 ms) at the end of the depolarizing pulses. For the V_{thr} determination, leak correction was performed using the first 5 (pH_e 7.4), 7 (pH_e 6.4 and 6.2), and 10 peak currents of the I-V relationship (i.e., between −80 and −50/−30/+10 mV) and the SD was calculated using the first 5 values in the I-V (i.e., between −80 and −50 mV). The V_{thr} was selected as the membrane potential at which the current was above 2 × SD.

For recording tail currents, the H_v1 current was fully activated using 500 ms long single-step depolarizations from a holding potential of −80 mV to +100 mV. The tail currents were recorded upon stepping back from this potential in 20 mV decrements to −60 mV, and the currents were recorded for 250 ms at the back-step potentials. The protocol

was applied every 15 s with a sampling rate of 20 kHz. The traces were leak-corrected manually and filtered (lowpass boxcar, 25 smoothing points). To test the presence of voltage-gated K^+ currents, 15-ms-long depolarization steps were applied to +50 mV from a holding potential of −100 mV every 15 s, with a sampling rate of 20 kHz. Voltage ramps, as specified above, were also used to study the presence of voltage-gated ion currents in MDSCs over an extended membrane potential range and depolarization duration in physiological salt solutions.

3.4.2 Recording whole-cell KCa3.1 currents in peripheral blood lymphocytes and transfected CHO cells

The cells were held at −85 mV holding potential to minimize the holding current and allow the calculation of the leak conductance. 150-ms-long voltage ramps (from −120 to +50 mV, in 150 ms) were applied every 10 s to evoke the $K_{Ca}3.1$ currents.

For offline leak correction and the calculation of the K^+ conductance a Python-based custom written program was used. The algorithm averaged the holding current (I_{hold}) for n=600 data points at –85 mV, which is close to the K^+ equilibrium potential calculated from the Nernst equation (E_K= –89 mV). I_{hold} was considered as leak current and used to calculate G_{leak} as I_{hold}/–85 mV. Every data point was corrected for linear leak using $I_K=I_m - (G_{leak} \times E_m)$, where I_K is the leak subtracted K^+ current, I_m is the measured membrane current at E_m membrane potential. I_K was then displayed as a function of E_m during the ramp and the region for linear current-voltage relationship was selected and G_K was calculated as the slope of the straight line fitted to the I_K data points ($G_K = \Delta I_K/\Delta V$). The linear region was typically between –120 mV and –60 mV for lymphocytes, at more depolarized membrane potentials the activation of the $K_v1.3$ current caused a significant deflection from the linear I-V relationship.

The following parameters were derived from the leak subtracted G_K data:

$G_{K,200}/G_{K,0}$: ratio of the K^+ conductances determined at a time point t >200 s ($G_{K,200}$) in standard extracellular solution (S-ECS) over G_K at the beginning of the experiment (0 s) in S-ECS ($G_{K,0}$) in the absence of $K_{Ca}3.1$ activators;

$G_{K,x}/G_{K,7.4}$: normalized conductance, G_K values determined in various pH_e solutions ($G_{K,x}$, where x is the pH_e) in a given cell normalized to $G_{K,7.4}$ recorded at pH_e=7.4 in the same cell;

$G_{K,act}/G_K$: “fold increase in conductance”, K^+ conductance measured in the presence of the activator at a given concentration ($G_{K,act}$) divided by the K^+ conductance in the drug-free solution (G_K)

“fold increase normalized to 7.4”: “fold increase in conductance” caused by the activator at a given pH_i and pH_e combination normalized to the “fold increase in conductance” measured with the activators in S- ECS at pH_e=7.4.

$G_{K,end}/G_{K,start}$: $G_{K,end}$ and $G_{K,start}$ are the activator-enhanced K^+ conductance at the end of the experiment (≥ 800 s) and the activator-enhanced K^+ conductance at the beginning of the experiment, respectively.

The activator concentration-response curves were fit using:

$$\text{fold increase in conductance} = \text{Bottom} + \frac{(Top-Bottom)\times[X]^{n_H}}{[X]^{n_H}+EC_{50}{}^{n_H}}$$

where "fold increase in conductance" is defined above, [X] is the activator concentration, EC_{50} is the concentration of agonist that gives a response halfway between Bottom (min value of "fold increase in the conductance") and Top (maximum value of the "fold increase in conductance"), and n_H is the Hill slope.

3.4.3 Data acquisition and analysis tools

The pClamp 10.5, 10.7, and 11.2 software packages were used to acquire the data. The pClamp 10.7 and 11.1 software packages (Molecular Devices Inc., Sunnyvale, CA, USA) were used to analyze the data. Statistical analyses were performed with GraphPad Prism 8.4.3 (GraphPad Software, Inc., San Diego, CA, USA).

3.5 Solutions

The standard extracellular solution used to study H_v1 at $pH_e = 7.4$ contained 180 mM HEPES, 75 mM N-Methyl-D-Glucamine (NMDG), 15 mM glucose, and 3 mM $MgCl_2$ (titrated with CsOH), whereas in the extracellular solutions at pH_e = 6.4/6.2/5.7, 180 mM HEPES buffer was substituted with 180 mM MES (2-(N-morpholino)ethanesulfonic acid, titrated with CsOH or HCl). The standard intracellular solution at pH_i = 6.2 contained 180 mM MES, 75 mM NMDG, 15 mM glucose, 3 mM $MgCl_2$, and 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, titrated with CsOH). To explore the presence of other voltage-gated currents, we used a Na^+-based extracellular solution at pH_e = 7.35 containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5.5 mM glucose, and 10 mM HEPES and a K^+-based intracellular solution at pH_i = 7.22 containing 140 mM KF, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, and 11 mM EGTA. The pH of the solutions was checked before every experiment, and all salts and components of the solutions were purchased from Sigma-Aldrich Budapest, Hungary.

The S-ECS used to study K_{Ca} channels was a Na^+-aspartate (Na^+Asp^-)-based solution with 2.5 mM $CaCl_2$, and 10 mM HEPES titrated to pH_e=7.4 with NaOH. The extracellular solution having pH_e=8.0 (8.0-ECS) was buffered with 10 mM HEPES whereas those having pH_e=6.0 (6.0-ECS); pH_e=6.5 (6.5-ECS) or pH_e=6.9 (6.9-ECS) were buffered using 10 mM MES, pH was titrated to the desired value with NaOH. The standard intracellular solution (S-ICS) was a K^+-aspartate (K^+Asp^-)-based solution with 8.5 mM $CaCl_2$, 10 mM EGTA and 10 mM HEPES (titrated to pH_i=7.2 with Tris). This solution has

an estimated free Ca^{2+} concentration of ~1-2 μM based on the MaxChelator program WEBMAX-C software (C. Patton, Stanford University, retrieved here: https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/webmaxc/webmaxcE.htm). Since pH has a strong effect on the affinity of EGTA for Ca^{2+} we substituted EGTA with BAPTA, a less pH-sensitive Ca^{2+} chelator, when the pH of the intracellular solution was titrated to various levels. To keep the free Ca^{2+} concentration around ~1-2 μM the pipette-filing solution titrated to pH_i=6.5 (6.5-ICS) contained 11 mM BAPTA whereas the one titrated to pH_i=8.0 (8.0-ICS) contained 10 mM BAPTA. For the experiments in which we used the K_{Ca}3.1 activators at pH=7.2 we set the free Ca^{2+} concentration in the pipette-filing solution to ~250 nM (7.2-ICS-250) and Ca^{2+} was buffered using EGTA (10 mM EGTA and 5.7 mM $CaCl_2$). When the pH of the pipette-filling solution, with 250 nM Ca^{2+} concentration, was set to pH_i=6.5 (6.5-ICS-250) or pH_i=8.0 (8.0-ICS-250) the amount of BAPTA was set to 10 mM, but the concentration of $CaCl_2$ was adjusted to reflect the pH-dependence of the buffer capacity of BAPTA. More precisely, in case of pH_i=6.5 we used 3.25 mM $CaCl_2$ and for pH_i=8.0 we used 4.3 mM $CaCl_2$. All pipette-filling solutions were titrated with Tris. All titrations were done at 25°C and the pH of solutions was checked before every experiment.

4. RESULTS

4.1 The voltage-gated H_v1 H^+ channel is expressed in tumor-infiltrating myeloid-derived suppressor cells

Myeloid-derived suppressor cells are a large component of the tumor milieu promoting tumor invasion and metastasis through several mechanisms. Even if the tumor microenvironment is very dynamic and ever-changing, it is usually characterized by hypoxia, nutrient poverty and acidity, features that do not seem to hinder MDSCs, which are able to adapt their metabolism to these extreme conditions, survive and act as pro-tumor agents. As a fact, tumor-derived lactic acid has been directly linked to positive regulation of MDSCs activity in murine pancreatic cancer. In this section, I will explain how we obtained fresh murine MDSCs, how we characterized them, and determined their ion channel repertoire that may provide a tool for pH adaptation.

4.1.1 MDSCs are a large component of Lewis lung cancer

LLC, a murine model representing non-small cell lung carcinoma, is recognized for harboring a minimal count of $CD4^+$ and $CD8^+$ T lymphocytes, while boasting a significant presence of myeloid cells such as macrophages, neutrophils, and dendritic cells. This characteristic renders it immunologically classified as a 'cold' tumor. MDSCs were isolated from LLC-tumor-bearing mice according to Materials and Methods and MDSC subfractions were sorted using flow cytometry.

Myeloid cells were identified using a combination of morphology, singlet, and viability gates in combination with the myeloid marker $CD11b^+$. In order to differentiate between the main two sub-populations (polymorphonuclear MDSCs and monocytic MDSCs) we used the markers Ly6G and Ly6C: PMN-MDSCs were defined as $CD11^+/Ly6G^+$ cells, whereas Mo-MDSCs as $CD11b^+/Ly6G^-/F4/80^-/MHCII^-/Ly6C^+$ cells. These two populations were not present with the same proportions: PMN-MDSCs were more abundant (60% of $CD11^+$ cells) and Mo-MDSCs less abundant (10% of $CD11^+$ cells). Since these cell surface markers do not unequivocally identify MDSC sub-populations, but are also expressed by other immune cell types, the only way to define these cells as MDSCs is to carry out T cell proliferation suppression assays, defined by the specialists as the “gold standard” technique. We showed that Mo-MDSCs had suppressive capacity when they were co-cultured with murine splenocytes in 1:1 and 1:2 ratios, whereas PMN-MDCSs failed to demonstrate immunosuppressive properties. Mo-MDSCs could suppress the proliferation of both $CD4^+$ and $CD8^+$ splenocytes. Interestingly, it is common to other tumor models that the scarce population of Mo-MDSCs is also one of the most immunosuppressive, while the abundant PMN-MDSCs show less or no immunosuppressive activity.

4.1.2 Both tumor-derived Mo- and PMN-MDSCs express H_v1 at mRNA and protein level

Recently it has been shown that *in vitro* differentiated murine MDSCs express H_v1. Inspired by this observation, we conducted qPCR to examine the expression of the H_v1 transcript in PMN- and Mo-MDSCs obtained from the LLC tumor. Although both MDSC subpopulations showed H_v1 gene expression, we observed a higher level of expression in PMN-MDSCs compared to Mo-MDSCs. This pattern was consistent when we analyzed H_v1 protein levels using Western blotting (WB). To gain more insight into the *in situ* expression of H_v1 in MDSCs infiltrating the LLC tumors, we used the H_v1-specific antibody, validated in WB analysis, to detect the H_v1 signal in tumor sections. The fluorescence signal was much stronger in PMN-MDSCs compared to Mo-MDSCs. PMN-MDSCs in the tumor showed focal distribution whereas the Mo-DSCSs are more sparsely distributed.

4.1.3 Ion currents in MDSCs

MDSCs acquired by cell sorting from the tumor mass underwent analysis via single-cell electrophysiology (patch-clamp) to assess the expression of whole-cell ion currents utilizing different combinations of intra- and extracellular solutions. Initially, we employed intracellular solutions based on K^+ and extracellular solutions based on Na^+, enabling the recording of voltage-gated K^+ and Na^+ currents. When either Mo- or PMN-MDSCs were subjected to 15 ms-long voltage steps from −100 mV holding potential to +50 mV test potential, we could not detect any classical voltage-gated ion currents in the outward direction. On the other hand, using voltage ramps, we detected a voltage-gated outward current that activated at depolarized membrane potentials and was sensitive to the extracellular pH. The presence of the proton current was observed more clearly when the recording solutions lacked conventional permeating cations and contained reduced Cl^- concentration to eliminate outward currents other than the proton current (using NMDG-based solutions) and rich in non-volatile buffers in order to keep both pH_i and pH_e stable.

4.1.3.1 Voltage-ramp analysis of the whole-cell H^+ currents in MDSCs

Voltage-ramp-evoked currents were recorded while the pH_i was maintained at 6.2 and the extracellular pH was changed to different values ranging from pH_e=5.7 to pH_e=7.4. The acidic pH_i was chosen for the recordings to mimic intracellular acidity that may happen in the TME. Moreover, this also facilitated the recordings as generation of the pH gradient across the membrane required to activate H_v1 could be achieved even at normal extracellular pH value. Our recordings show several features characteristic of H_v1. First, the smaller the pH gradient across the membrane [ΔpH_{e-i} = (pH_e − pH_i)] the more depolarized the V_{thr} (threshold voltage). V_{thr} was estimated in voltage-ramp experiments by the membrane potential at which the whole cell current deviated from leak. The large difference in the V_{thr} values as a function of pH_e is clearly demonstrated for both types of MDSCs. Second, the larger the pH gradient, the larger the currents are at identical

membrane potentials. Plotting the current of each sweep at +145 mV as a function of the sequentially numbered sweeps shows that changing the extracellular pH induces rapid and reversible effects on the current amplitude. The capacitance measurements suggest that Mo-MDSCs are bigger than PMN-MDSCs (1.83 ± 0.14 (n = 40) vs. 3.13 ± 0.14 pF (n = 39), mean ± SEM, $p < 0.0001$), the H^+ current density in PMN-MDSCs was ~3 times bigger compared to Mo-MDSCs at $pH_e = 7.4$.

4.1.3.2 Voltage-step analysis of the whole-cell H^+ currents in MDSCs and the quantitative determination of the threshold potential for activation

The V_{thr} shift can be quantitatively inferred from the current–voltage relationships. The families of whole-cell currents were obtained in a single PMN-MDSC upon applying 2000 ms-long step voltage depolarizations in 10 mV increments. The intracellular pH was maintained at pH_i=6.2 and the extracellular pH ranged from pH_e=7.4 to pH_e=5.7 with 6.4 and 6.2 as intermediate values. While larger depolarizations resulted in currents with quicker activation kinetics, 2 s-long pulses did not suffice to achieve current saturation. This phenomenon is commonly observed in H_v1 currents. The same experiments, with the same extra- and intracellular pH, were done on Mo-MDSCs.

For both PMN-MDCs and Mo-MDSCs, the V_{thr} values are shifted to more depolarized membrane potentials as pH_e became more acidic while keeping the intracellular pH at $pH_i = 6.2$. The values of V_{thr} for PMN-MDSCs were −16.7 ± 3.1 mV at $\Delta pH_{e-i} = 1.2$; +20 ± 5 mV at $\Delta pH_{e-i} = 0.2$; +15.0 ± 6.5 mV at $\Delta pH_{e-i} = 0$; and +63.3 ± 3.3 mV at $\Delta pH_{e-i} = -0.5$. For Mo-MDSCs, the V_{thr} was −14.4 ± 5.5 mV for $\Delta pH_{e-i} = 1.2$; +40.0 ± 10.8 mV at $\Delta pH_{e-i} = 0.2$, +38.0 ± 9.2 mV for $\Delta pH_{e-i} = 0$; and +57.5 ± 7.5 mV at $\Delta pH_{e-i} = -0.5$.

The linear regression analysis of the V_{thr}-ΔpH_{e-i} relationship did not deviate from the "rule of forty", i.e., (~40 mV shift per one unit ΔpH change) for either PMN- or Mo-MDSCs, however, the V_{thr}-ΔpH_{e-i} is shifted to depolarized potentials for the currents recorded in Mo-MDSCs.

4.1.3.3 H^+ selectivity and pharmacology of the whole-cell H^+ currents in MDSCs

The H^+ selectivity of currents in PMN-MDSCs was assessed by deriving the reversal potential (E_{rev}) through analyzing whole-cell tail currents. Cells were depolarized to +100 mV for 500 ms to initiate the current, succeeded by repolarizations to diverse membrane potentials (ranging from −60 to +100 mV) to elicit the tail currents. The membrane potential where the polarity of the tail current reversed was defined as the reversal potential. The gradient of the best-fit linear regression line illustrates that E_{rev} shifts by −42 mV for each one-unit alteration in the extracellular pH. Nevertheless, this slope differs from that of a perfectly selective H^+ conductance (−59.16 mV per one unit ΔpH change) as predicted by the Nernst equation. The currents in Mo-MDSCs were too minimal to allow for a reliable tail current analysis.

The literature frequently relies on ClGBI as a pharmacological criterion for identifying H_v1 currents. It is evident that 200 μM ClGBI almost entirely inhibited the whole-cell current in a PMN-MDSC, and this inhibition was reversible as the current reverted to its original level after rinsing the recording chamber with ClGBI-free extracellular solution. The onset of inhibition was rapid, whereas it required more than 30 cycles in a ClGBI-free solution to wash out the effect. Contrary to PMN-MDSCs, we were able to do pharmacological experiments in Mo-MDSCs only using repeated application of a voltage-ramp protocol where long exposure to depolarized test potentials can be avoided. Regardless of the voltage protocol used (i.e., step depolarization vs. voltage ramp), the application of 200 μM ClGBI reduced the magnitude of the current significantly, ~80% reduction in PMN-MDSCs and 75% in Mo-MDSCs.

4.2 Intracellular acidity impedes $K_{Ca}3.1$ activation by Riluzole and SKA-31

The tumor microenvironment is a complex system inhabited by pro- and anti-tumor cells whose fine balance is easily disrupted. We have seen that pro-tumor MDSCs rely on H_v1 as their main ion channel and H_v1 is characterized by a strong sensitivity to pH. On the other end, anti-tumor infiltrating T cells rely on $K_v1.3$ and $K_{Ca}3.1$ to exert their function. Virtually nothing is known about the influence of pH on $K_{Ca}3.1$ and on its positive modulators. In this section I will explain how we studied the effect of pH on $K_{Ca}3.1$ in two different expression systems and the effect of pH on two modulators of the aforementioned channel, Riluzole and SKA-31.

4.2.1 Extracellular pH variations minimally influence K^+ currents through $K_{Ca}3.1$ expressed endogenously in PBLs

The expression of $K_{Ca}3.1$ is transcriptionally up-regulated when T cells are stimulated with mitogens. We took advantage of this and induced T cell proliferation with phytohemagglutinin A (PHA) to enhance $K_{Ca}3.1$ expression. Alongside PHA activation of T cells, we supplemented the pipette filling solution with a Ca^{2+} (1 μM concentration) to ensure complete activation of the $K_{Ca}3.1$ current. PHA treatment also increases the expression of the voltage-gated $K_v1.3$ K^+ channel in T cells. Isolated $K_{Ca}3.1$ currents in activated T cells can be recorded by either pharmacological separation, achieved through the inhibition of $K_v1.3$ current using peptide toxins, or by analyzing whole-cell currents below the activation threshold of $K_v1.3$. We opted for the latter approach. The threshold for the activation of $K_v1.3$ typically falls between −30 and −40 mV, although this can be cell- and recording condition-dependent and some authors extend the range to −60 mV to −50 mV. Moreover, V_{thr} for $K_v1.3$ activation is sensitive to changes in pH_e: acidification of the extracellular pH causes a shift of V_{thr} towards higher values. Because of all these reasons, we have assigned the currents to $K_{Ca}3.1$ in the region between −120 mV and −60 mV to avoid contamination of the analysis of $K_{Ca}3.1$ currents by a $K_v1.3$ component. The current-voltage relationship is linear within this range, therefore, the slopes of the straight lines

fitted to this section of the traces were utilized to ascertain the $K_{Ca}3.1$-specific K^+ conductance, denoted as G_K thereafter (calculated as $G_K = \Delta I/\Delta V$). The reversal potential (E_{rev}) of the leak-corrected currents falls within the range of −75 mV to −100 mV, indicating the K^+ selectivity of the current. The theoretical E_{rev} of a K^+ selective conductance, calculated from the Nernst equation, is E_K= −89 mV. $K_{Ca}3.1$ currents recorded at various pH_e values ranging from pH_e=8.0 to pH_e=6.0 overlap when the S-ICS (pH=7.2) was employed as the pipette filling solution. Changing pH_e alters minimally but fully and readily reversibly G_K. The same set of experiments were repeated using pipette-filling solution having pH_i=8.0 (8.0-ICS) and pH_i=6.5 (6.5-ICS). The results were essentially the same as at pH_i=7.2: at pHi=7.2 and pHi=8.0 there is a slight yet noteworthy reduction in the normalized conductance occurred with the transition to acidic extracellular pH values, while the G_K remained essentially unchanged when the pH_i was 6.5, irrespective of variations in pH_e. Moreover, G_K values at pH_e=7.4 are relatively constant throughout a given experiment, regardless of the pH_i. Taking the ratio of the K^+ conductances determined at a time point t >200 s ($G_{K,200}$) in S-ECS over G_K at the beginning of the experiment (0 s) in S-ECS ($G_{K,0}$) resulted in $G_{K,200}/G_{K,0}$ ~1 thereby indicating the stability of the whole-cell $hK_{Ca}3.1$ currents.

4.2.2 Extracellular pH variations minimally influence K^+ currents through $K_{Ca}3.1$ expressed heterologously in CHO cells

We repeated the same set of experiments using cells transfected with the $hK_{Ca}3.1$ gene. We decided to use the commonly used epithelial Chinese Hamster Ovary (CHO) cell line since these cells do not express $K_v1.3$ or other K_v channels, therefore one can minimize the contamination of the data by other K^+ conductances. However, since the channels are overexpressed in this cell model, we observed currents reaching several nA of amplitude, which is several folds greater than what we observed in PBLs at identical intracellular Ca^{2+} concentration. Pharmacological data obtained using overexpressed ion channels due to the increased $K_{Ca}3.1$ conductance are more reliable since the effect of leak on the whole-cell K^+ conductance is minimized. Similarly to human lymphocytes, $K_{Ca}3.1$ currents recorded at pH_e values ranging from pH_e=8.0 to pH_e=6.0 in CHO cells are superimposable when the S-ICS (pH=7.2) was used as the pipette filling solution. Changing pH_e alters G_K minimally but fully and readily reversibly. The same set of experiments were repeated using pipette-filling solution having pH_i=8.0 (8.0-ICS) and pH_i=6.5 (6.5-ICS). Just like in PBLs, G_K values remain relatively constant at pH_e=7.4 across the duration of an experiment, irrespective of the pH_i, indicating the stability of the whole-cell $hK_{Ca}3.1$ currents.

4.2.3 Normal and basic intracellular pH do not interfere with activation of the $K_{Ca}3.1$ current either by Riluzole or by SKA-31

Riluzole and its more potent derivative SKA-31 are positive modulators of the $K_{Ca}3.1$ currents in micro- and nanomolar-range concentrations, respectively. To determine the experimental EC_{50} values, i.e. the concentrations at which the substances cause half

maximal activation of the current, we applied increasing concentrations of Riluzole or SKA-31 to transfected CHO cells and measured G_K. When we use an intracellular concentration of 1 μM Ca^{2+} the currents through $K_{Ca}3.1$ are already saturated, hence a pharmacological enhancement cannot be fully appreciated. Given that the enhancement of the $K_{Ca}3.1$ current by the activators is more prominent at lower cytosolic Ca^{2+} concentrations compared to the ~1 μM Ca^{2+} concentration utilized previously, we employed pipette filling solutions containing 250 nM Ca^{2+} concentration (7.2-ICS-250) in this series of experiments. The G_K values under this condition gradually increased with the concentration of SKA-31 or Riluzole. To construct the concentration-response curve, we calculated the fold increase in conductance caused by the activators as suggested in the literature and plotted this variable as a function of the activator concentration. The EC_{50} values for Riluzole and SKA-31 were, respectively, 6.0 ± 1.3 μM and 570 ± 101 nM as calculated by averaging the EC_{50} values determined individually cell-by-cell.

To examine the influence of extracellular pH on the effects of $K_{Ca}3.1$ activators, Riluzole (5 μM) or SKA-31 (1 μM) were introduced into extracellular solutions spanning a pH_e range from 6.0 to 8.0. The activator concentrations were chosen to be close to the EC_{50} of the substances. Meanwhile, the intracellular pH of whole-cell patch-clamped PBLs was adjusted and buffered at pH_i=7.4, pH_i=8.0, or pH_i=6.5 using the suitable intracellular solution. The raw current traces produced by voltage ramps indicate that the baseline $K_{Ca}3.1$ currents are nearly negligible. Upon perfusing the recording chamber with the activators in the S-ECS the $K_{Ca}3.1$ conductance is instantaneously increased regardless of the extracellular solution's pH_e and the removal of activators reduces the current within 2-4 sweeps, corresponding to 20-40 s. The reversible effect of the activators can be repeatedly observed over prolonged periods with no significant decline in $K_{Ca}3.1$ conductance. To quantify this we utilized the $G_{K,end}/G_{K,start}$ ratio, which resulted in values around 1, meaning that G_K values remain relatively constant across the duration of an experiment. Similar outcomes were observed when using the 8.0-ICS-250 pipette filling solution. To compare the impact of the activators, we initially computed the "fold increase in conductance" induced by the activator at specific pH_i and pH_e combinations, then normalized it to the "fold increase in conductance" observed with the activators in S-ECS at pH_e=7.4. The resulting "fold increase normalized to 7.4" values, obtained in this manner, consistently clustered around 1 for both Riluzole and SKA-31, regardless of the pH_i (7.2 or 8.0) or the pH_e (8.0, 6.9, 6.5, or 6.0) meaning that the two substances have the same effect regardless of intra- and extracellular pH.

We have shown that the enhancements of the $K_{Ca}3.1$ current by Riluzole and SKA-31 exhibit similar characteristics under identical experimental conditions. Consequently, we focused our subsequent experiments on SKA-31, which is more potent and selective. We replicated the same series of experiments in CHO cells expressing $K_{Ca}3.1$. The current in the absence of SKA-31 increases robustly upon perfusing the cells with solutions of different pH_e and supplemented with 1 μM SKA-31. The currents

recorded in various pH_e values are superimposable, i.e., the potency of SKA-31 was the same regardless of the pH_e. The characteristics of the enhancement of the $K_{Ca}3.1$ current by SKA-31 remained consistent throughout the experiment when the activator was administered repeatedly to a cell. A qualitatively similar phenomenon was recorded when the cell was patch-clamped using an 8-ICS-250 pipette filling solution. It is important to emphasize here that G_K was repeatedly activated by SKA-31 to the same extent at both pH_i values over an extended period of time following the start of the activator application. The reversible effect of the activators can be repeatedly observed over prolonged periods with no significant decline in $K_{Ca}3.1$ conductance ($G_{K,end}/G_{K,start}$ ratio ~1) with both pH_i=7.2 and pH_i=8.0 pipette filling solutions. The variable "fold increase normalized to 7.4" shows values clustering around 1, mirroring the observations made for PBLs.

4.2.4 Activation of the KCa3.1 current either by Riluzole or by SKA-31 gradually decreases in time when the intracellular pH is acidic

The behavior of the $K_{Ca}3.1$ current in response to Riluzole and SKA-31 in PBLs changed drastically when the pipette-filling solution was 6.5-ICS-250, corresponding to a pH_i of 6.5. The effectiveness of both Riluzole and SKA-31 gradually diminishes with prolonged application of either drug. To characterize this decline in potency over time, we utilized the $G_{K,end}/G_{K,start}$ ratio. The decline in conductance was consistent and this phenomenon was unaffected by the pH_e: in other words, it continued unabated across all pH_e values until reaching saturation at approximately 20% of the conductance elicited by the activator at the beginning of the experiment.

Similarly to the findings in PBLs, the $K_{Ca}3.1$ conductance gradually diminished with repeated administration of SKA-31 to the same transfected CHO cell at pH_i=6.5 (6.5-ICS-250). The gradual decline of SKA-31-induced conductance persisted at a seemingly constant rate regardless of the pH_e of the extracellular solution. The K^+ conductance remained unrecoverable upon re-administration of SKA-31 in the control S-ECS solution with pH_e=7.4. This loss of potency phenomenon was also evident when the S-ECS while pH_i was slightly more alkaline (pH_i=6.7) or more acidic (pH_i=6.2). The loss of the K^+ conductance over the time-course of the experiment was characterized quantitatively using the $G_{K,end}/G_{K,start}$ ratio (median of 0.17).

To gain deeper insights into the nature of the loss-of-potency phenomenon, we investigated the response of the currents to SKA-31 at a constant pH_e of 7.4 (S-ECS) and pH_i of 6.2 while varying the pattern of SKA-31 application. The first experimental “pattern” protocol involved SKA-31 administration at the onset of the experiment, followed by a washout phase. Subsequently, the cell underwent repeated depolarizations using voltage ramps every 10 seconds for 400 seconds in the absence of SKA-31. After this interval, SKA-31 was reapplied, yet it failed to potentiate the current to the same extent as observed during its initial application to this cell.

The $G_{K,end}/G_{K,start}$ ratio is significantly below 1, indicating a decrease in the potentiation of K^+ conductance by the end of the experiments. Consequently, the loss-of-potency phenotype persists despite the introduction of a drug-free period into the protocol, during which voltage ramps are repeatedly applied. In the second special "pattern" protocol, the $K_{Ca}3.1$ conductance was initially potentiated by SKA-31 application. Following this, while the solution containing SKA-31 was continuously perfused onto the cell, the delivery of voltage ramps was halted for 360 seconds, and the cell was maintained at a holding potential of –85 mV during this interval. Subsequently, the voltage ramps were resumed. The SKA-31-induced $K_{Ca}3.1$ conductance was considerably reduced after the interruption in recording compared to the beginning of the experiment. The $G_{K,end}/G_{K,start}$ ratio is markedly below 1, unequivocally affirming the loss-of-potency phenotype by the conclusion of the experiments. Consequently, neither the incorporation of a voltage-ramp-free period nor a drug-free period into the protocol can forestall the decline in the potency of SKA-31. This suggests that the loss-of-potency phenotype must be ascribed to the acidic pH_i.

4.2.5 $K_{Ca}2.2$ activation is sensitive to intracellular pH_i similarly to $K_{Ca}3.1$

$K_{Ca}2.x$ channels, akin to $K_{Ca}3.1$, are triggered by intracellular Ca^{2+} and respond to the same modulators. However, $K_{Ca}2.x$ channels exhibit approximately tenfold lower sensitivity to compounds such as Riluzole and SKA-31. We selected $K_{Ca}2.2$ as the representative channel from this family and evaluated the efficacy of SKA-31 at a higher concentration of 5 μM in activating the current under neutral and acidic intracellular pH conditions. At a pH_i of 7.2, robust activation of $K_{Ca}2.2$ by SKA-31 could be consistently induced with minimal decline in the modulator's effectiveness over time. Conversely, when the intracellular solution was adjusted to pH_i=6.5, a rapid and irreversible decline in the potency of SKA-31 to activate $K_{Ca}2.2$ was observed, mirroring the findings observed for $K_{Ca}3.1$.

4.2.6 Mutations H192A in $hK_{Ca}3.1$ and T79D in CaM do not influence the loss-of-potency phenotype at acidic intracellular pH

Protonation of histidine residues in acidic environments can disrupt the ability of toxins to bind to ion channels, making the toxins less or non-functional. The recent cryo-EM-derived structure of $hK_{Ca}3.1$ unveiled the involvement of the S4-S5 linker in establishing both the functional and structural connection between Calmodulin and the C-terminal portion of the K_{Ca} channels. His192 residue, situated within this linker, is positioned near the pocket where activators are believed to exert their influence. Substituting this histidine with a non-charged alanine (H192A) led to the disruption of the interaction between BA6b9, a $K_{Ca}3.1$ blocker designed to share structural similarities with Riluzole, and $K_{Ca}3.1$.

Given the structural resemblance between BA6b9 and the modulators utilized in this investigation, along with their compatibility with overlapping binding sites, we

examined whether the H192A mutation would impact the activation of $hK_{Ca}3.1$ by SKA 31 under both neutral and acidic conditions. SKA-31 activated the H192A-$K_{Ca}3.1$ current in a reversible manner, akin to wild-type $K_{Ca}3.1$. The efficacy of SKA-31 in activating the current remained relatively stable over prolonged durations. However, at pH_i 6.5, SKA-31 gradually lost its effectiveness in activating the H192A-$K_{Ca}3.1$ current upon repeated administration. This indicates that the loss of SKA-31-mediated potentiation of the current at acidic intracellular pH is independent of whether the protonated His or the neutral Ala is present at position 192.

Calmodulin (CaM) maintains constitutive binding to K_{Ca} channels, requiring membrane-bound PIP_2 as a co-agonist in addition to Ca^{2+} ions. Thr79 within CaM serves as a substrate for Casein Kinase-2 (CK2). Phosphorylation of Thr79 results in diminished sensitivity of the $K_{Ca}2.2$ channel to both PIP_2 and Ca^{2+}. This phosphorylation can be mimicked by the phosphomimetic mutation T79D, which decreases K^+ current in both $K_{Ca}2.2$ and $K_{Ca}3.1$. The altered network of activators and co-activators in the presence of T79D-CaM may impact the modulation of $K_{Ca}3.1$ activation by SKA-31 under acidic pH_i conditions. To investigate this, we co-transfected $hK_{Ca}3.1$ and T79D-CaM into CHO cells and assessed the potentiation of the whole-cell current at neutral and acidic pH_i. Under neutral pH_i conditions, the current could be activated by SKA-31 similar to cells transfected with $hK_{Ca}3.1$ only. Activation cycles by SKA-31 resulted in consistent increases in K^+ conductance over extended periods. Conversely, when pH_i=6.5 was employed, SKA-31 gradually lost its potency over time in activating G_K.

Although the average loss of G_K by the experiment's end (>800 s) was slightly reduced, the $G_{K,end}/G_{K,start}$ ratios were similar when wild-type-$hK_{Ca}3.1$, H192A-$hK_{Ca}3.1$ or wild-type-$hK_{Ca}3.1$ and T79D-CaM were transfected into CHO. Additionally, some cells exhibited a very slow restoration of SKA-31 potency over time. This phenomenon was not further investigated due to the inherent limitations of whole-cell patch-clamp techniques over prolonged durations exceeding 15-20 minutes.

4.2.7 High intracellular Ca^{2+} concentration hinders the inhibitory effect of intracellular acidity

$K_{Ca}3.1$ is extremely sensitive to intracellular calcium concentrations, with an EC_{50} ranging from 100 to 400 nM and a typical sigmoidal activation curve. When SKA-31 was administered to $hK_{Ca}3.1$-expressing CHO cells and a 1 μM Ca^{2+} concentration was used in the pipette at a pH_i of 7.2, we observed: 1) an increased baseline $K_{Ca}3.1$ conductance due to the elevated intracellular calcium levels and 2) a reduced potentiation of G_K by SKA-31 (approximately 2-fold compared to approximately 50-fold at a cytosolic Ca^{2+} concentration of 250 nM), attributable to the near-saturation levels of channel calcium sensitivity. Intriguingly, at a cytosolic Ca^{2+} concentration of 1 μM, the effectiveness of SKA-31 in enhancing $K_{Ca}3.1$ conductance was maintained at acidic pH_i=6.5. The $G_{K,end}/G_{K,start}$ parameter obtained at pH_i=6.5 and 1 μM Ca^{2+} did not show statistically

significant differences compared to the data obtained at pH_i=7.2 at either 1 μM or 250 nM cytosolic Ca^{2+} concentration. This suggests that at saturating intracellular calcium concentrations the efficacy of SKA-31 in activating KCa3.1 remains constant regardless of the pH_i.

5. DISCUSSION

5.1 The voltage-gated H_v1 H^+ channel is expressed in tumor-infiltrating myeloid-derived suppressor cells

A key novelty of our study is that the expression of H_v1 was shown in MDSCs obtained from tumors. Our findings revealed that tumor-derived PMN-MDSCs were the most prevalent subset in LLC tumors, detected by flow cytometry approximately six times more frequently than Mo-MDSCs. This prevalence is consistent with similar tumor types, and comparable proportions have been reported in pancreatic ductal adenocarcinoma (PDAC) and autoimmune diseases such as autoimmune arthritis.

Due to the complexity of defining MDSCs solely based on membrane markers, it is a common practice to confirm the identity of these cells through functional studies. Typically, this involves demonstrating their ability to suppress T cell proliferation to avoid confusion with phenotypically similar monocytes and neutrophils. In our investigation, we found that tumor-derived Mo-MDSCs exhibited T-cell suppressive activity, while PMN-MDSCs, despite their higher abundance, did not demonstrate such suppression, at least under the MDSC/splenocyte ratios utilized in our experiments. This lack of anti-proliferative behavior in PMN-MDSCs aligns with findings from other studies conducted in LLC tumors in mice and has also been observed in contexts such as PDAC, autoimmune arthritis, and MDSCs found in transplanted organs in humans. However, PMN-MDSCs may still promote tumor growth by inhibiting cytolytic T cell activation directly and indirectly influencing other myeloid cells and NK cells. They are known to be the primary source of immunosuppressive mediators like ROS and RNS, which dampen TCR signaling and regulate cytokine secretion. Furthermore, PMN-MDSCs hinder the recruitment of cytolytic T cells and contribute to tumor progression by releasing matrix metalloproteinases (MMPs) and factors that facilitate tumor angiogenesis. Moreover, a recent electrophysiological study provides strong evidence that the cells classified as PMN-MDSCs in our study are distinct from neutrophils. Immler and colleagues demonstrated through electrophysiological assays that neutrophil polymorphonuclear leukocytes (PMN) functionally express voltage-gated $K_v1.3$ K^+ channels, a feature notably absent in our whole-cell recordings of MDSCs, where $K_v1.3$ or any other voltage-gated K^+ current was not detected.

Several lines of evidence support that whole-cell H_v1 currents were recorded in tumor-derived Mo- and PMN-MDSCs in our study. First, the currents were slowly activating, rapidly deactivating, and with no sign of inactivation, which is characteristic of

H_v1. Moreover, the currents were recorded using intra- and extracellular solutions that lacked (K^+, Na^+) or contained negligible concentration (Cl^-) of conventional permeating ions; thus, the contribution of other conductances to the whole-cell current, that could mimic the behavior of H_v1, are minimized.

Second, the whole-cell currents observed in both types of MDSCs were influenced by the pH gradient across the membrane and the membrane potential. The threshold voltage required for the activation of these currents shifted along the voltage axis in response to changes in ΔpH, approximately 40 mV per unit change in extracellular pH, closely resembling previous descriptions of proton currents in various cell types, including bone marrow-derived MDSCs. Additionally, we observed that the V_{thr} in PMN-MDSCs was depolarized compared to Mo-MDSCs under symmetrical pH conditions ($pH_e \sim pH_i$), although this did not affect the overall V_{thr}–ΔpH relationship of approximately 40 mV per one unit ΔpH change. The V_{thr} of approximately +40 mV in symmetric solutions in PMN-MDSCs is qualitatively similar to that determined for the H_v1 current in murine neutrophil granulocytes (approximately +50 mV), which are closely related to PMN-MDSCs. However, it remains uncertain whether the difference between the two cell types we observed stem from technical errors primarily due to the extremely low ion currents in Mo-MDSCs or from translational or post-translational differences between H_v1 in PMN- and Mo-MDSCs.

Third, the H_v1 current observed in PMN-MDSCs exhibits a notable selectivity for H^+, as evidenced by the E_{rev}-ΔpH relationship closely resembling the theoretical relationship predicted for H^+ ions from the Nernst equation. The small currents observed in MDSCs are susceptible to contamination by non-specific leakage, even with applied leak corrections; any contribution of leakage to the whole-cell current shifts the reversal potentials towards depolarized potentials. The challenges arising from incomplete leakage subtraction precluded the reliable determination of the reversal potential in Mo-MDSCs, where currents are exceedingly small, often less than 100 pA even under optimal ΔpH and membrane potential conditions.

Fourth, the currents observed in both PMN-MDSCs and Mo-MDSCs demonstrated sensitivity to ClGBI, a guanidine derivative small molecule inhibitor of H_v1 and commonly utilized as an indicator of H_v1 current presence in various cell types.

Fifth, the electrophysiological findings are strongly corroborated by molecular biology analyses, where the mRNA transcript of H_v1 was identified in MDSCs using RT-qPCR, along with the H_v1 protein itself being detected in Western blots. The human H_v1 proton channel protein exhibits two isoforms: a long, full-length isoform and a shorter variant lacking an N-terminal region due to alternative splicing. The antibody utilized in our study recognizes both isoforms, a fact confirmed in the CH12 mouse B cell lymphoma cell line used as a positive control in our investigation, as well as in human B cell lymphomas reported previously. However, we observed only the long form in both PMN-

MDCS and Mo-MDSCs isolated from the LLC tumor. The absence of the short form suggests that it may not be expressed or may be present in negligible amounts below the detection limit, indicating that the long isoform of Hv1 likely contributes to the functionality of tumor-derived MDSCs.

While the H_v1 proton channel has been extensively characterized in various immune cell types, to our knowledge, there is a lack of information regarding H_v1 expression in tumor-associated inflammatory cells. Our study, for the first time, identified a substantial population of H_v1^+ myeloid cells within a tumor, consistent with the cell surface marker phenotype of PMN- and Mo-MDSCs. Our electrophysiology findings are in line with data reported for in vitro-generated MDSCs, where H_v1 currents of comparable magnitude (ranging between 200 pA and 1 nA at +130 mV) were observed using the patch-clamp technique in a mixed MDSC population generated through the differentiation of murine bone marrow-derived myeloid precursors induced by GM-CSF. Our findings suggest that in vitro-differentiated MDSCs could serve as valuable tools for investigating H_v1-dependent regulation of T cell function in cancer, given the similarity in channel phenotype between these cells and tumor-derived MDSCs regarding H_v1 expression. Additionally, we demonstrated that both PMN- and Mo-MDSCs exhibit H_v1-mediated H^+ currents, albeit to varying degrees, indicating that the ion channel phenotype of the two MDSC subtypes is comparable, at least in mice.

What could be the functional implications of H_v1-mediated H^+ currents in MDSCs? Neutrophils, which share close similarities with MDSCs, express functional H_v1, and these H^+ currents play a role in balancing positive charge efflux necessary for sustaining ROS production. Given that ROS production is also a hallmark of MDSCs' immunosuppressive activity, the functional expression of H_v1 in MDSCs and its susceptibility to H_v1 inhibitors appears reasonable. Consistent with this notion, electrophysiological studies have demonstrated H_v1-mediated H^+ currents in MDSCs and blocking H_v1 using inhibitors like ClGBI and Zn^{2+} has been shown to inhibit ROS production and alleviate the suppression of T cell proliferation by MDSCs. However, prolonged exposure of MDSCs to H_v1 inhibitors for more than 2 hours resulted in significant cell death, raising concerns about the specificity of ClGBI's effects.

The proton efflux facilitated by the H_v1 proton channel might additionally contribute to the acidic environment within the TME, a condition that tumor cells can tolerate well but which hinders the tumor-suppressive capabilities of T cells and NK cells. Consequently, regulating the acidity of the tumor microenvironment through H_v1 inhibition could potentially enhance the anti-tumor actions of immune cells in cancer therapy. However, recent findings have also indicated that the heightened intracellular acidity in activated T cells resulting from the absence of the H_v1 proton channel diminishes the effector function of these T cells. This aspect must also be considered when evaluating the overall efficacy of H_v1-targeted cancer therapy. Moreover, changes in the extracellular and intracellular pH may influence the operation of the ion channels of immune cells, cancer

cells and other cellular components of the TME. As ion channels in cancer cells and cellular components of the TME are attractive pharmacological targets to treat cancer, it bears outmost importance to determine the effectiveness of ion channel modulators under the very special intra- and extracellular pH and ionic milieu characteristic for the TME.

5.2 Intracellular acidity impedes $K_{Ca}3.1$ activation by Riluzole and SKA-31

To our knowledge, we delivered the first comprehensive study that analyzes how extra- and intracellular pH influences the magnitude of the $hK_{Ca}3.1$ current and its potentiation by the positive modulators of the channel SKA-31 and Riluzole. We showed that the $hK_{Ca}3.1$ current expressed endogenously in human peripheral blood lymphocytes or expressed heterologously in CHO cells shows very subtle sensitivity to the pH_i ranging from 6.5 to 8.0 and pH_e ranging from 6.0 to 8.0. The very potent activators of $K_{Ca}3.1$, Riluzole and SKA-31, induce robust $K_{Ca}3.1$ currents at normal (pH_i = 7.2) and alkaline (pH_i = 8.0) intracellular pH for both endogenously and heterologously expressed channels. On the other hand, the potency of SKA-31 in activating the $K_{Ca}3.1$ current declines over time when the intracellular pH is acidic (pH_i < 6.5). The loss of the potency of SKA-31 was not specific for $K_{Ca}3.1$, the potentiation of the current also declined over time when $K_{Ca}2.2$ was studied at pH_i = 6.5. The loss of the SKA-31 potency at acidic pH_i was also shown for a $K_{Ca}3.1$ mutant where a titratable His was mutated to Ala (H192A) near the binding pocket for the activators. Similarly, transfection of CHO with T79D, a Calmodulin mutant that confers reduced Ca^{2+} sensitivity to $K_{Ca}3.1$, did not prevent the loss-of-potency phenotype when SKA-31 was applied at acidic pH_i. However, increasing the cytosolic Ca^{2+} concentration to 1 µM eliminated the loss-of-potency phenotype of SKA-31 activation at acidic pH_i.

The reliance of K^+ conductance on extracellular pH likely stems from multiple factors. One possibility is associated with the alteration of surface charges. Unlike voltage-gated channels, $K_{Ca}3.1$ lacks the charges in the S4 helix of the VSD characteristic for other voltage-gated ion channels. Hence, the absence of an effect of pH_e on K^+ conductance in $K_{Ca}3.1$ is not unexpected. Additionally, pH_e can regulate ion channels by specifically interacting with exposed titratable amino acid residues. Notably, acidic extracellular pH can significantly influence the conductance, inactivation kinetics, and pharmacology of $K_v1.3$ due to the presence of a titratable His residue in the channel's pore entrance. However, the human $K_{Ca}3.1$ channel contains a valine at a corresponding position (V257). While there are titratable amino acid residues near the selectivity filter of $K_{Ca}3.1$, such as H236 and D239, and even if these become protonated at acidic pH_e, they do not notably affect the K^+ conductance of $K_{Ca}3.1$ channels. Acidic pH_e notably decreases K^+ currents through $hK_{Ca}3.1$, although the reduction in current never exceeds 15%–20% compared to pH_e = 7.4. Moreover, this effect is primarily observed at extracellular pH 6.0, which is exceedingly low and may represent the lower extreme in pathological context.

Based on the lack of sensitivity of the $K_{Ca}3.1$ current to variations in both intracellular and extracellular pH, we infer that the gating mechanism of $K_{Ca}3.1$ and the network of co-activators (Ca^{2+}, CaM, and PIP_2) remain unaffected by pH levels relevant to both physiological and pathophysiological conditions. This conclusion appears to contradict earlier research that demonstrated pH sensitivity in the shape, Ca^{2+} binding capacity, and Ca^{2+} affinity of CaM. These contrasting outcomes may stem from previous studies utilizing isolated CaM in solution or employing mathematical models, which could account for the discrepancy with our findings.

A notable pharmacological property of $K_{Ca}3.1$ is its responsiveness to a group of small molecules which act as channel activators. The mechanism of action of these activators involves shifting the calcium-activation curve towards lower intracellular Ca^{2+} concentrations in a concentration-dependent manner, consequently increasing the apparent Ca^{2+} affinity. However, they are incapable of activating the channels in the absence of intracellular Ca^{2+}. Thus, while they function as positive-gating modulators, they also exhibit a super-agonist effect. Both SKA-31 and Riluzole enhance the $K_{Ca}3.1$ current across all combinations of pH_e-pH_i, as long as the intracellular pH remains neutral or basic.

In contrast, when the intracellular pH was acidic, both SKA-31 and Riluzole demonstrated a decline in their ability to activate $K_{Ca}3.1$ over the duration of several hundred seconds in our experiments. One possible explanation for this loss of potency could be that exposure to acidic intracellular pH induces an irreversible structural change in the activator molecules. However, considering the structures of Riluzole and SKA-31 and their predicted pK_a values (2.96 and 3.5), their protonation status is minimally affected within the pH range of 6.0 to 8.0. Both Riluzole and SKA-31 maintained their effectiveness when dissolved throughout the day in an extracellular solution with a pH of 6.0. Furthermore, upon immediate application of SKA-31 or Riluzole, the $K_{Ca}3.1$ current was enhanced even under acidic intracellular pH conditions. Additionally, upon exposing the intracellular environment to acidic pH, the decline in SKA-31-mediated current activation progressed when we paused SKA-31 application or interrupted the current recordings for several hundred seconds. The only intervention that prevented this loss of potency was the increase in cytosolic Ca^{2+} concentration to 1 μM.

Another possibility for the reduction in $K_{Ca}3.1$ conductance induced by the activators at acidic intracellular pH could include the acidic intracellular pH, low (250 nM) Ca^{2+} concentration, and the presence of activators, which could result in decreased availability of the channels for opening.

Pharmacologically activating $K_{Ca}3.1$ using positive modulators has emerged as a promising strategy to enhance the suppressed immune response against cancer. This approach holds particular significance for overcoming the immunosuppressive TME. Given that the intracellular pH in the acidic TME is also acidic, the benefits of $K_{Ca}3.1$ positive modulators may be compromised by the loss-of-potency phenotype observed at

acidic intracellular pH in our study. On the other hand, various cancer types exhibit $K_{Ca}3.1$ overexpression. In these cases, using an activator could potentially be counterproductive. Therefore, the overall impact of the acidic intracellular pH-induced loss of $K_{Ca}3.1$ activator potency must be carefully assessed for both immune system function and cancer cell behavior.

6. SUMMARY

The tumor microenvironment includes diverse immune cell types immersed in a very complex and hostile system characterized by hypoxia, nutrient deficiency and acidity. Acidity, in particular, will directly affect the functionality of several proteins, among which ion channels. In this PhD dissertation we tested whether $K_{Ca}3.1$, an ion channel essential for lymphocytic activation and proliferation, is affected by changes in extracellular and intracellular pH. We discovered that K^+ currents through $K_{Ca}3.1$ were minimally, but reversibly, altered by a shift in the extracellular pH. Notably, acidic pH_e caused a current drop and basic pH_e caused a current increase. These changes were statistically significant, but never extended beyond ~20% compared to the baseline and could probably be considered physiologically irrelevant. We also enquired into the pH sensitivity of two $K_{Ca}3.1$ activators: Riluzole and SKA-31. These substances have been suggested as possible oncological immune boosters, therefore their relationship with pH should be better characterized. We showed that both Riluzole and SKA-31 activate $K_{Ca}3.1$ in a fast and reversible manner, unfazed by alkaline or acidic extracellular pH. However, when the intracellular pH was shifted to acidic values (6.2-6.7) both Riluzole and SKA-31 lost their potency in a time-dependent and an irreversible manner. We were not able to determine which molecular components contributed to this phenomenon, since mutations of sensitive spots in $K_{Ca}3.1$ (H192A) and in the ion channel-bound Calmodulin (T79D) did not restore the lost potency. However, a high Ca^{2+} intracellular concentration apparently restabilizes the optimal functionality of SKA-31, suggesting a possible role of Calmodulin in this newly discovered phenomenon.

Lymphocytes are not the only immune cells inhabiting the tumor microenvironment. Another important cell type is the myeloid-derived suppressor cell, which has been shown to host H_v1 as its main ion channel. H_v1 is the only H^+ channel in mammals and it is strongly sensitive to changes in pH and $\Delta pH_{e\text{-}i}$. However, most of the information we have about H_v1 in myeloid-derived suppressor cells stem from *in vitro*-generated murine cells, which can be different from *in vivo* ones. We obtained tumor-related myeloid-derived suppressor cells after subcutaneously implanting a tumor mass in the flank of C57BL/10 mice and analyzed the expression of H_v1 through Western blotting, qPCR, immunofluorescence and patch clamping. Through patch clamping we showed that these cells do not apparently host other voltage-gated currents except for the H^+ current, which was identified thanks to several biophysical and pharmacological pieces of evidence.

7. PUBLICATIONS

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Subject: PhD Publication List

Candidate: Marco Cozzolino
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Cozzolino, M.**, Panyi, G.: Intracellular acidity impedes KCa3.1 activation by Riluzole and SKA-31.
   Front. Pharmacol. 15, 1-23, 2024.
   DOI: http://dx.doi.org/10.3389/fphar.2024.1380655
   IF: 5.6 (2022)
2. **Cozzolino, M.**, Gyöngyösi, A., Korpos, É., Gogolák, P., Naseem, M. U., Kállai, J., Lányi, Á., Panyi, G.: The Voltage-Gated Hv1 H^+ Channel Is Expressed in Tumor-Infiltrating Myeloid-Derived Suppressor Cells.
   Int. J. Mol. Sci. 24 (7), 1-24, 2023.
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   IF: 5.6 (2022)

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List of other publications

3. Somodi, L., Bekéné Debreceni, I., Kis, G., **Cozzolino, M.**, Kappelmayer, J., Antal, M., Panyi, G., Bárdos, H., Mutch, N. J., Muszbek, L.: Activation mechanism dependent surface exposure of cellular factor XIII on activated platelets and platelet microparticles.
   J. Thromb. Haemost. 20 (5), 1223-1235, 2022.
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   Front. Pharmacol. 11, 1-28, 2021.
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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